# Chemotherapy regulated microRNA-125/HER2 pathway as a novel therapeutic target for trastuzumab-mediated cellular cytotoxicity in small-cell lung cancer.

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**Abstract:**

Small-cell lung cancer (SCLC) accounts for 15% of all lung cancer cases and is a highly lethal disease. For the last several decades, the standard treatment for SCLC has been deadlocked, and new therapeutic strategies are urgently needed. Human epidermal growth factor receptor 2 (HER2) is a member of the HER family and has been reported to be overexpressed in 30% of SCLC cases with poor prognosis. However, the clinical relevance of HER2-targeted therapy for SCLC remains unclear. Here, we firstly identify that cytotoxic drugs induce significant HER2 overexpression through microRNA-125a (miR-125a) and miR-125b downregulation, which in turn act as a novel therapeutic target for trastuzumab-mediated cellular cytotoxicity in SCLC. In this study, we showed that treatment of the HER2-positive SCLC cells, SBC-3 and SBC-5, with cytotoxic drugs induced a significant upregulation of HER2. Cisplatin (CDDP) treatment of SCLC cells resulted in a significant downregulation of miR-125a and miR-125b. We confirmed that miR-125a and miR-125b bound to the 3'-untranslated regions of HER2 mRNA, and that downregulation of miR-125a and miR-125b resulted in upregulation of HER2 in SCLC cells, suggesting a relationship between cytotoxic drug exposure and miR-125/HER2 dysregulation. Furthermore, using a calcein assay we demonstrated a significantly enhanced cytotoxic effect of CDDP and trastuzumab that was mediated via antibody-dependent cellular cytotoxicity. Finally, we clearly demonstrated the enhanced anti-tumor effect of these agents

in an orthotopic lung cancer model *in vivo*. Our result offer a novel therapeutic strategy for

HER2-positive SCLC by using trastuzumab combined with cytotoxic drugs.

**Introduction**

Lung cancer is the leading cause of cancer related deaths all over the world. Small-cell lung cancer (SCLC) accounts for 15% of all lung cancer cases (1). Approximately 70% of all newly diagnosed SCLC patients present with advanced disease and require systemic chemotherapy. Despite a dramatic response to front-line chemotherapy, drug-resistance is the fundamental issues that cannot be avoided, and SCLC is still an inevitably fatal disease. The recent development of molecular targeted agents for epidermal growth factor receptor (EGFR) activating mutations and anaplastic lymphoma kinase (ALK) translocation have greatly changed the therapeutic strategy for, and have prolonged survival time in non-small cell lung cancer (NSCLC) (2, 3). In the meantime, the therapeutic strategy for SCLC has remained as platinum-based combination chemotherapy for the last several decades (4). Although a few recent SCLC sequencing studies have reported novel putative driver gene alterations, specific molecular targeting therapy against such alteration is not yet available (5-7) .

Human epidermal growth factor receptor 2, also known as HER2 or erbB2/*neu*, is a member of the erbB receptor tyrosine kinase family. HER2, which is encoded by the ERBB2 gene, activates cellular proliferation and survival signals mainly as a heterodimer with other HER family members (8-10). HER2 has been reported to be overexpressed in 30% of breast cancers and in 20-30% of gastric cancers, correlating with their poor prognosis (11, 12).

Similarly, HER2 is reported to be overexpressed in approximately 30% of SCLCs and is known to be a negative prognostic factor for SCLC (13-15). Trastuzumab, a monoclonal anti-HER2 antibody, has been approved for the treatment of HER2-positive breast and gastric cancers and is a widely used indispensable drug. Despite the importance of trastuzumab combined with standard chemotherapy for the treatment of HER2-positive breast and gastric cancers, the precise mechanisms of their enhanced effect remain unknown. Moreover, the efficacy of trastuzumab alone or in combination with standard chemotherapy for SCLC has yet to be clarified.

MicroRNAs (miRNAs), a family of short endogenous noncoding RNAs, act as posttranscriptional gene regulators through binding their target messenger RNAs (mRNA) (16). It was recently shown that miRNA-125a-5p (miR-125a) and its homolog miRNA-125b (miR-125b) directly regulate HER2 in human breast cancer cells (17). Likewise, miR-125a expression has been shown to have prognostic significance for gastric cancer (18). The impact of miR-125a or miR-125b on HER2 expression in SCLC remains unknown.

Based on the above data, we hypothesized that cytotoxic drugs might boost HER2 expression via changes in miR-125a or miR-125b expression, and that this upregulated HER2 expression could be targeted by trastuzumab for SCLC treatment. In this study, we demonstrated that exposure of HER2-positive SCLC cells to cytotoxic drugs resulted in

downregulation of miR-125a and miR-125b, and accordingly HER2 overexpression. Notably, the combination therapy of trastuzumab and CDDP showed an enhanced anti-tumor effect *in vitro* that was mediated via antibody-dependent cellular cytotoxicity (ADCC). This enhanced effect was confirmed using an orthotopic lung cancer model *in vivo*. These data suggest the importance of ADCC in trastuzumab-mediated antitumor activity. They further suggest that cytotoxic drug-induced regulation of the miR-125/HER2 pathway is one of the hidden mechanisms behind the enhanced anti-tumor effect of trastuzumab and cytotoxic drugs and that this mode of cytotoxicity involves ADCC. Our study offers a novel therapeutic strategy for HER2-positive SCLC by combination therapy with trastuzumab and cytotoxic drugs.

**Materials and Methods**

**Cell culture.** The human SCLC cell lines H69, H82, H187, and H446 (purchased in 2013), the human breast cancer cell lines SK-BR-3 and MCF-7 (purchased in 2000), and the NK cell line NK92 (purchased in 2012) were purchased from the American Type Culture Collection (Rockville, MD). The SBC-3 and SBC-5 cell lines were obtained from Okayama University in 1994. All SCLC cell lines were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO), SK-BR-3 cells in McCOY's 5a (Sigma-Aldrich), MCF-7 cells in Minimum Essential Medium Eagle (Sigma-Aldrich), and NK92 cells in Myelocult H5100 medium (STEMCELL technologies, Vancouver, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and an antibiotic-antimycotic (Invitrogen, Carlsbad, CA). NK92/CD16a, a previously reported NK-92 cell line that stably expresses CD16, was cultured in Myelocult H5100 medium supplemented with 10% FBS and 1% geneticin (10131-027; Gibco, Carlsbad, CA) (19). All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$. All cell lines were authenticated by short tandem repeat profiling and compared with the known ATCC database.

**Reagents.** Rabbit monoclonal anti-HER2 (#4290), anti-phospho-HER2 (#2247), anti-HER3 (#12708), anti-phospho-HER3 (#4561), anti-EGF receptor (#2232), anti-Akt (#4685), anti-phospho-Akt (#4060), anti-Erk (#4695), and anti-phospho-Erk (#4370) were obtained from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti-HER2/neu APC

(340554) and mouse monoclonal IgG1 K isotype control APC antibody (554681) was obtained from BD Biosciences (Franklin Lakes, NJ). Synthetic hsa-miR-125a-5p (pre-miR-125a-5p; PM12561, anti-miR-125a-5p; PM12561), hsa-miR-125b-5p (pre-miR-125b-5p; PM10148, anti-miR-125b-5p; PM10148), pre-miR miRNA precursor molecules Negative Control (PM17110), and anti-miR miRNA Inhibitor Negative Control (PM17010) were obtained from Applied Biosystems (Foster City, CA). Cisplatin (CDDP) and irinotecan (CPT-11) were obtained from Yakult Honsha Co., Ltd. (Tokyo, Japan). Etoposide (ETP) was obtained from Nippon Kayaku Co., Ltd. (Tokyo, Japan). Trastuzumab was obtained from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

**Cell viability assay.** Cells were plated in 96-well plates and were then treated with various concentrations of CDDP on the following day. Cell viability was determined at the indicated time using the WST-8 assay (cell counting Kit-8; Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. The absorbance at 450 nm was measured. Cell viability was expressed as a percentage of that of the control (untreated) cells. For each concentration of each drug, mean values of the mean absorbance rates from six wells were calculated. The $IC_{50}$ (concentration of drug needed to inhibit cell growth by 50%) was generated from dose-response curves for each cell line (Supplementary Table 1). All experiments were performed as three independent repeated experiments.

**Immunoblot analysis.** Cells were seeded and were grown overnight. The cells were then washed with ice-cold phosphate buffered saline (PBS) and lysed with M-PER (Thermo Scientific, San Jose, CA) supplemented with protease inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitors (Sigma-Aldrich). Cell lysates were separated by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to a PVDF membrane. SDS-PAGE gels were calibrated using Magic Mark XP Western Standard (LC5602, Invitrogen). Primary antibodies were used at a dilution of 1:1000. Secondary antibodies (peroxidase-labelled anti-mouse and anti-rabbit antibodies) were used at a dilution of 1:2000. Bound antibodies were visualized by chemiluminescence using the ECL Prime Western blotting detection system (RPN2232, GE Healthcare, UK), and luminescent images were analyzed with a Lumino Imager (LAS-4000 mini; Fuji Film Inc., Tokyo, Japan).

**Flow cytometry.** Cells were suspended in their culture medium and FCM analysis was performed using a Cell Analyzer EC800 (SONY Biotechnology Inc., Tokyo, Japan). At least one million cells were pelleted by centrifugation at 300 x $g$ for 5 min at 4°C, resuspended in a 5 μL of a monoclonal mouse anti-HER2-APC antibody (BD Bioscience) or a monoclonal mouse IgG1 κ isotype control APC antibody (BD Bioscience) dilution, and incubated for 15 minutes at 4°C.

**RNA extraction, reverse transcription and quantitative real-time RT-PCR.** Total RNA was extracted from cultured cells using QIAzol and the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purity and concentration of all RNA samples were quantified using NanoDrop 2000 (Thermo Scientific). The qRT-PCR method has been previously described (20). PCR was performed in 96-well plates using Step One Plus (Applied Biosystems). All reactions were performed in triplicate. Hsa-miR-125a-5p, hsa-miR-125b-5p, and endogenous control RNU6B TaqMan microRNA assays were obtained from Applied Biosystems. SYBR Green qRT-PCR was performed for qRT-PCR of messenger RNA, and the β-actin housekeeping gene was used to normalize the variation in the cDNA levels. All primer sequences are listed in Supplementary Table 2.

**Transient transfection.** MiRNA (25 nmol/l) was transfected using the Lipofectamine RNAiMAX reagent (Invitrogen), according to the manufacturer's protocol. Vector plasmid transfection (500 ng) was carried out using the Lipofectamine LTX reagent (Invitrogen), according to the manufacturer's protocol. Vector plasmid combined with each miRNA mimic transfections were carried out using the DharmaFECT Duo reagent (Thermo Scientific), according to the manufacturer's protocol.

**Plasmids.** The LightSwitch 3' untranslated region (3'-UTR) reporter GoClone for ERBB2 (S805256, ERBB2-Luc w/t) was obtained from SwitchGear Genomics (CA, USA) for the

3'UTR assay. To make miR-125 binding site mutants, the 3'-UTR of HER2 (sequence: CUCAGGG) was mutated to the sequence CACTGCG, using the KOD –Plus- Mutagenesis Kit (SMK-101, TOYOBO, Tokyo, Japan), according to the manufacturer's protocol. The mutated vector (ERBB2-Luc mut) was confirmed by sequencing. For luciferase-based *in vivo* assays, pLucNeo was constructed by inserting a firefly luciferase gene derived from the pGL3-control (Promega, Madison, WI) into the pEYFP-1 vector (Clontech Laboratories, Mountain View, CA) at BglII and AflII sites, as previously described(21).

**3'UTR assay.** Cells were seeded in 96-well plates at a density of 8,000 (SBC-3) to 10,000 (SBC-5) cells per well the day before transfection. The cells were collected 48 hr after co-transfection with either ERBB2-Luc w/t or the ERBB2-Luc mut and either pre-miR-125a-5p or pre-miR-125b-5p. Luciferase activity was then measured using the LightSwitch Assay Reagent (SwitchGear Genomics), according to the manufacturer's protocol.

**Establishment of a cell line stably expressing luciferase.** For establishment of a cell line stably expressing luciferase, the SBC-5 cell line, at 80% confluency in 24-well dishes, was transfected with pLuc-Neo (500 ng) using the Lipofectamine LTX reagent, according to the manufacturer's protocol. After 24 h incubation, the cells were replated into a 15 cm dish

followed by 3-week selection with 1,500 ng/ml geneticin. This cell line was termed SBC-5-Luc.

**ADCC assay.** ADCC was examined using a calcein-acetyoxymethyl (Calcein-AM; Dojindo) release assay. The target cells were labeled with Calcein-AM for 30 min, then washed and plated onto 96-well plates at a density of 5,000 cells/well in triplicate. Trastuzumab was added at various concentrations ranging from 0.00001 to 10 μg/ml, and then NK92/CD16a cells were added as effector cells at an effector: target (E:T) ratio of 2.5:1, 5:1 or 10:1. The plates were then incubated for 4 hr at 37 °C and calcein release (cell death) into the supernatants was analyzed using fluorometry. For maximum release, the cells were lysed with 0.1% Nonidet P-40 (Roche) prior to assay. The fluorescence value of the culture medium background was subtracted from that of the experimental release (A), the target cell spontaneous release (B), and the target cell maximal release (C). Percentages of cytotoxicity and ADCC were calculated using the following formulae:

Cytotoxicity (%) = (A - B) / (C - B) x 100

ADCC (%) = Cytotoxicity (%, with trastuzumab) – Cytotoxicity (%, without trastuzumab).

To estimate the ADCC activity of trastuzumab with or without CDDP, each target cell was treated with or without an IC50 concentration of CDDP for 48 hr. Next, target cells were

trypsinized and dead cells were washed away to exclude the cytotoxicity of CDDP during 48 hr of exposure. Remaining viable cells were labeled with calcein-AM for 30 min, then plated onto 96-well plates at a density of 5,000 cells/well in triplicate. In this experiment, for the purpose of excluding the cytotoxicity of CDDP during 4 hr of calcein assay even washed away before assay, the spontaneous release of CDDP treated cells (dead cells during 4 hr) were subtracted from the experimental release of trastuzumab with CDDP treated cells.

**Animal studies.** Animal experiments were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. Five- to six-week-old male C.B-17/Icr-scid/scid Jcl mice (CLEA, Tokyo, Japan) were used in the experiments. Intrathoracic injections were performed following a previous protocol with minor modifications (22). Briefly, the cells were resuspended in PBS at half the inoculum volume and were diluted with an equal volume of growth factor-reduced Matrigel (356231; Corning Inc., Corning, NY). Mice anesthetized with sodium pentobarbital (50 mg/kg body weight) were placed in the right lateral decubitus position. One-ml tuberculin syringes with 30-gauge hypodermic needles were used to inject the cell inoculum percutaneously into the left lateral thorax, at the lateral dorsal axillary line, ~1.5 cm above the lower rib line just below the inferior border of the scapula. The needle was quickly advanced 5-7 mm into the thorax and was rapidly removed after injection of the cell suspension. After tumor injection, the mouse was turned to the left lateral decubitus position. Animals were observed for 45–60

min until fully recovered. For *in vivo* imaging, D-luciferin (150 mg/kg) was administered to the mice (Wako, Osaka, Japan) by intraperitoneal injection. Ten minutes later, photons from the whole bodies of the animals were counted by measuring bioluminescence with an IVIS imaging system (Xenogen, Alameda, CA), according to the manufacturer's instructions. The data were analysed using LIVINGIMAGE 4.2 software (Xenogen). The development of lung cancer was monitored twice a week *in vivo* using bioluminescent imaging. Bioluminescence from the implanted cancer cells was measured one week after tumor injection (day 7), and the mice were then divided into four treatment groups with equivalent levels of bioluminescence. Each treatment: —3 mg/kg of CDDP alone, 20 mg/kg of trastuzumab alone or 3 mg/kg of CDDP plus 20 mg/kg of trastuzumab —was performed on days 7, 14, 21, and 28 (once a week for 4 weeks, four treatments in total). All mice were sacrificed on day 42 and the presence of the tumor was histologically confirmed. Mice without confirmation of lung tumor were excluded from the analysis. As a result, the final total number of each group was as follows: control (n=4), CDDP alone (n=7), trastuzumab alone (n=5) and CDDP plus trastuzumab (n=7).

**Statistical analysis.** All *in vitro* experiments were repeated at least three times. The results are expressed as means ± SD. The statistical analyses were mainly conducted using Student's t-test for *in vitro* experiments and two sample Student's t-tests for *in vivo*

experiments. All the analyses were performed using STATA version 12.0 (Stata Corp,

College Station, TX). Probability values of <0.05 indicated a statistically significant difference.

**Results**

**HER2 expression in various SCLC cell lines.** To assess HER2 protein expression in SCLC, we examined various SCLC cell lines by western blotting analysis (WB). Of the six SCLC cell lines analyzed, only SBC-3 and SBC-5 cells showed obvious HER2 expression (Figure 1A). WB analysis of other members of the HER family indicated that HER3 was expressed in 4 of these cell lines but that the EGFR was only expressed in the SBC-3 cell line. We also analyzed HER2 expression on the surface of these cell lines using flow cytometry (FACS, Figure 1B). This FACS analysis confirmed the WB result that, of the SCLC cell lines tested, only SBC-3 and SBC-5 cell lines expressed HER2.

**HER2 expression is upregulated by exposure of SCLC cells to cytotoxic drugs.** We next assessed HER2 expression change after exposure of all six cell lines to cytotoxic drugs in clinical use for SCLC. We first analyzed HER2 expression change by WB after exposure of SCLC cell lines to CDDP, the key drug for SCLC treatment in clinical practice. The protein expressions of HER2 in SBC-3 and SBC-5 cells were upregulated in a time dependent manner after CDDP exposure for up to 24hr. In contrast, even exposure to various CDDP concentrations and exposure times did not result in HER2 expression in the HER2-negative cell lines (H69, H82, H187 and H446) (Supplementary Figure 1A). We then confirmed the CDDP-induced HER2 expression change in SBC-3 and SBC-5 cells by mRNA, WB, and

FACS analysis following exposure to $IC_{50}$ of CDDP for up to 48 hr (Figure 2A, B, C). The $IC_{50}$ of CDDP for each of these cell lines is shown in Supplementary Table 1. HER2 expression was highest by all methods of analysis after 48 hr exposure of the cells to CDDP (Figure 2A, B, C). Next, we assessed the effect of exposure of the SBC-3 and SBC-5 cells to other cytotoxic drugs, namely etposide (ETP) and irinotecan (CPT-11), on HER2 expression by WB. Upregulation of HER2 expression was also observed following 48 hr exposure to CPT-11 and, to some extent, to ETP (Supplementary Figure 1B).

**CDDP treatment of cells induces the dysregulation of microRNA-125a and microRNA-125b.** To investigate the unknown mechanisms of HER2 upregulation after exposure of the cells to cytotoxic drugs, we focused on miR-125a and miR-125b that are reported to directly regulate HER2 and HER3 expression in breast cancer cells (17). Using quantitative real time-polymerase chain reaction (qRT-PCR), we assessed the expression of miR-125a and miR-125b after exposure of SBC-3 and SBC-5 cells to the $IC_{50}$ dose of CDDP for up to 48 hr (Figure 2D). The levels of both miR-125a and miR-125b were significantly downregulated 24 hr and 48 hr after CDDP treatment compared to those of control cells.

**HER2 is directly regulated by microRNA-125a and microRNA-125b in SCLC.** To confirm the binding of miR-125a-5p and miR-125b to the 3'-UTR of the mRNA encoding HER2 in SCLC, a luciferase reporter assay was carried out using a vector that included the 3'-UTR

sequence of HER2 downstream from the luciferase reporter gene (ERBB2-Luc w/t) (Figure 3A). Transient co-transfection of the reporter plasmid and pre-miR-125a or pre-miR-125b into SBC-5 cells significantly reduced luciferase activity compared with the negative control (Figure 3B). In contrast, the luciferase activity of the reporter construct with a mutated 3'-UTR sequence (ERBB2-Luc mut) was unchanged by co-transfection with either pre-miR-125a or pre-miR-125b (Figure 3B). These data suggest that HER2 mRNA is a direct functional target of miR-125a and miR-125b in SCLC cells.

**Downregulation of microRNA-125a or microRNA-125b promotes HER2 overexpression.** To investigate the relationship between dysregulation of miR-125a or miR-125b and HER2 expression, we compared the effects of transient transfection of SBC-3 and SBC-5 cells with pre-miR-125a, pre-miR-125b, anti-miR-125a, or anti-miR-125b on HER2 mRNA and protein expression. As shown in Figure 3C, the expression of HER2 mRNA in SBC-5 cells did not change after transient transfection of anti-miR-125a and anti-miR-125b, whereas significant HER2 mRNA downregulation was observed after transfection of pre-miR-125a and pre-miR-125b. WB analysis indicated that HER2 protein expression was upregulated in anti-miR-125a and anti-miR-125b treated cells whereas it was downregulated in pre-miR-125a and pre-miR-125b treated cells (Figure 3D). These data suggest that downregulation of both miR-125a and miR-125b could directly induce HER2 upregulation. Moreover, these results may indicate that CDDP-induced miR-125

downregulation could be a novel mechanism of HER2 overexpression after cytotoxic chemotherapy.

**Growth inhibitory effect of CDDP and trastuzumab in the presence or absence of effector cells.** To investigate the growth inhibitory effect of trastuzumab and CDDP, including determination of whether this effect was direct or indirect, we used a WST-8 assay of cell viability in the presence or absence of effector cells. As shown in Figure 4A, 1 μg/ml of trastuzumab treatment of SBC-3 and SBC-5 cells resulted in little cytotoxicity in the absence of effector cells. In contrast, in the presence of effector cells at an E:T ratio of 1:1, trastuzumab alone exerted a significant cytotoxic activity against SBC-5 cells but not against SBC-3 cells, presumably because of the different expression level of HER2 in these cells. Interestingly, this trastuzumab-mediated cytotoxic activity was significantly enhanced by concurrent CDDP exposure. Moreover, we analyzed downstream signaling of HER2 including phospho-HER2, phospho-HER3, phospho-Akt, and phospho-Erk because these signaling pathways have been reported to be decrease after trastuzumab exposure in breast cancer cells (23) (Supplementary Figure 2). However, we could not find any contribution of this intracellular signaling in SBC-3 and SBC-5 cells. Phospho-HER2 and phospho-HER3 were negative in all of the time point or drug exposure setting of both SBC-3 and SBC-5 cells. Moreover, phospho-Akt and phospho-Erk were unchanged or at least not decreased after exposure to trastuzumab alone. We found downregulation of phospho-Akt and phospho-Erk

after exposure to CDDP and correspondingly upregulated cleaved PARP 48 hr after CDDP exposure. These results suggest that the trastuzumab-mediated growth inhibitory effect against SCLC cells is not a direct effect but an indirect effect that involves ADCC.

**Antibody-dependent cellular cytotoxicity in SCLC cell lines.** We next investigated the importance of ADCC for the indirect effect of trastuzumab on cell viability. For this purpose we assayed ADCC activity against SBC-3 and SBC-5 cells using a calcein-release assay. SK-BR-3 cells were used as a positive control. For all three cell lines, ADCC activity tended to increase in a trastuzumab dose-dependent and Effector: Target ratio (E:T ratio) dependent manner. Maximum ADCC activity was observed at a dose of trastuzumab of approximately 1 $\mu$g/ml and an E:T ratio of 10:1, regardless of the cell line (Figure 4B and 4C). Using the calcein-release assay we then examined the effect of exposure of these cells to combination treatment of CDDP for 48 hr followed by trastuzumab exposure on ADCC. The results show a statistically significant upregulation of ADCC activity against both SBC-3 and SBC-5 cells following sequential CDDP and trastuzumab treatment compared to cells treated with either agent alone (Figure 4D).

**Effect of combined CDDP and trastuzumab treatment in an orthotopic lung cancer model.** To examine the effect of combined CDDP and trastuzumab treatment against lung cancer *in vivo*, we established an SBC-5-Luc orthotopic lung cancer model, which we

monitored using bioluminescence imaging. One week after intrathoracic tumor injection (day 7), mice were treated with either PBS (control), trastuzumab (20 mg/kg) alone, CDDP (3 mg/kg) alone, or CDDP plus trastuzumab (Figure 5A). Following treatment once a week for four weeks, all of the drug-treated mice showed a significantly greater growth inhibitory effect towards the tumor compared to the control group. Treatment of mice with CDDP resulted in significantly higher growth inhibition than treatment with trastuzumab. Notably, treatment with CDDP plus trastuzumab resulted in a significantly greater growth inhibitory effect than treatment with CDDP alone or with trastuzumab alone (Figure 5B and 5C). These results indicate that combination therapy with trastuzumab and CDDP is a promising treatment strategy for HER2 positive SCLC.

**Discussion**

We showed that, of six SCLC cell lines examined, the SBC-3 and SBC-5 cell lines expressed HER2. These cell lines are adherent, do not express neural cell adhesion molecule (NCAM), and derived from Japanese SCLC patients. Several previous reports have shown that the percentage of HER2-positive SCLC clinical sample is approximately 30%, which compares favorably with the percentage of HER2-positive breast or gastric cancers. Since anti-HER2 treatment is a useful therapy for these other cancers, expression of HER2 in SCLC cells suggests the possibility that HER2 may also be a promising therapeutic target for SCLC (24, 25). Previously, Minami et al. found six HER2 positive SCLC cell lines among 13 SCLC cell lines including SBC-3 and SBC-5 and that all of these HER2 positive cell lines were derived from Japanese SCLC patients, indicating the ethnic differences of HER2 expression. Moreover, they also clearly demonstrated the HER2 increase in drug resistant cell lines (SBC-3/CDDP, SBC-3/ETP, and SBC-3/SN-38) compared with their parent cells (25). We, in this study, investigated HER2 expression change in SCLC cells, after treatment with the cytotoxic drugs CDDP, ETP and CPT-11 that are commonly used in clinical practice. We found that CDDP and CPT-11 induced upregulation of HER2 in HER2-positive SBC-3 and SBC-5 cells not in HER2-negative cells (H69, H82, H187, and H446).

In order to understand the mechanism of HER2 upregulation that we observed after drug exposure, our study focused on miR-125a and miR-125b. We clearly demonstrated the downregulation of miR-125a and miR-125b at 24 hr after exposure of SCLC to CDDP, and demonstrated that this downregulation was accompanied by HER2 upregulation. Moreover, we confirmed that HER2 is a direct target of both miR-125a and miR-125b in SCLC cells. These results suggest that cytotoxic drug-induced miR-125a and miR-125b downregulation might be a novel mechanism of HER2 upregulation after drug exposure.

In this study, we investigated the function of miR-125a and miR-125b for the regulation of HER2 in SCLC. In breast cancer cells, miR-125b has been reported to suppress the oncoproteins MUC1, HER2 and HER3, inhibiting the growth of breast cancer cells and promoting their sensitivity to chemotherapy (17, 26). Considering that miR-125/HER2 pathway may have a crucial role in chemoresistant SCLC, our results can also offer a possibility of miR-125 replacement therapy for preventing drug-resistance in HER2-positive SCLC.

We could not demonstrate the mechanism of miR-125a and miR-125b dysregulation after cytotoxic drug exposure. MiR-125, especially miR-125b, has been reported to be regulated by nuclear factor kappa beta (NF-κβ) depend on the cellular context (27, 28). Meanwhile, cytotoxic chemotherapies such as CDDP, ETP, and CPT-11 are well known for activating NF-κβ (29-31). In this context, although we could not demonstrate the significant

HER2 upregulation by treatment with ETP, we suppose that chemotherapy exposure first induces NF-$\kappa\beta$ activation and correspondingly downregulates miR-125a and miR-125b expression. Further investigation into the relationship between NF-$\kappa\beta$ and miR-125 in SCLC is needed.

In the near future, it should be possible to bring enhanced effect by a combination of CDDP and miR-125a or miR-125b replacement therapy. However, the technique of miRNA replacement therapy is not yet in general use. Therefore, we employed anti-HER2 antibody trastuzumab to target HER2 overexpression induced by cytotoxic drugs. Trastuzumab has been reported to exert antitumor activity through both direct and indirect activities (32). Of these various activities of trastuzumab, we and others previously showed that the induction of ADCC was important for antibody-induced antitumor activity (33-36). In ADCC, the cytotoxic effect depends on the subtype of fragment C $\gamma$ receptor (Fc$\gamma$R) expressed in immune effector cells and the expression level of each targeted membranous protein (37). In the present study, we confirmed that trastuzumab shows almost no growth inhibitory effect towards SCLC cells in the absence of effector cells (Figure 4A). To avoid the effect of Fc$\gamma$R subtypes in ADCC, we employed NK92/CD16a cells as effector cells for the WST-8 and calcein assay, as we previously described(36). By using this system of the WST-8 assay in the presence of effector cells, we demonstrated the enhanced effect of trastuzumab and CDDP and we further confirmed the induction of ADCC activity of the combined treatment by

using calcein-release assay. These results indicate the importance of ADCC for the trastuzumab-mediated cytotoxic effect and the relevance of trastuzumab therapy for HER2-positive SCLC cells following their exposure to cytotoxic drugs *in vitro.* These results could be also applied to understanding the enhanced effect of trastuzumab and cytotoxic drugs against breast or gastric cancer. Moreover, we confirmed the enhanced effect of a combination of trastuzumab and CDDP using an orthotopic lung cancer model that was monitored bioluminescence imaging. Using this model, we were able to reproduce tumor growth similar to that observed in the living human body.

In conclusion, we firstly identified a cytotoxic drug-induced miR-125/HER2 pathway as a novel mechanism underlying the enhanced effect of trastuzumab and cytotoxic drugs. Secondly, combination therapy of trastuzumab and a cytotoxic drug exerted an enhanced anti-tumor effect via ADCC *in vitro* and *in vivo*. Our result offer a novel therapeutic strategy for HER2-positive SCLC by using trastuzumab combined with cytotoxic drugs.

References

1.    van Meerbeeck JP, Fennell DA, De Ruysscher DK. Small-cell lung cancer. Lancet. 2011;378:1741-55.

2.    Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004;350:2129-39.

3.    Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature. 2007;448:561-6.

4.    Noda K, Nishiwaki Y, Kawahara M, Negoro S, Sugiura T, Yokoyama A, et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. N Engl J Med. 2002;346:85-91.

5.    Peifer M, Fernandez-Cuesta L, Sos ML, George J, Seidel D, Kasper LH, et al. Integrative genome analyses identify key somatic driver mutations of small-cell lung cancer. Nat Genet. 2012;44:1104-10.

6.    Rudin CM, Durinck S, Stawiski EW, Poirier JT, Modrusan Z, Shames DS, et al. Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. Nat Genet. 2012;44:1111-6.

7.    McFadden DG, Papagiannakopoulos T, Taylor-Weiner A, Stewart C, Carter SL, Cibulskis K, et al. Genetic and clonal dissection of murine small cell lung carcinoma progression by genome sequencing. Cell. 2014;156:1298-311.

8.    Cross M, Dexter TM. Growth factors in development, transformation, and tumorigenesis. Cell. 1991;64:271-80.

9.    Hung MC, Lau YK. Basic science of HER-2/neu: a review. Semin Oncol. 1999;26:51-9.

10.    Tebbutt N, Pedersen MW, Johns TG. Targeting the ERBB family in cancer: couples therapy. Nat Rev Cancer. 2013;13:663-73.

11.    Ross JS, Fletcher JA. The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. Stem Cells. 1998;16:413-28.

12.    Marx AH, Tharun L, Muth J, Dancau AM, Simon R, Yekebas E, et al. HER-2 amplification is highly homogenous in gastric cancer. Hum Pathol. 2009;40:769-77.

13.     Micke P, Hengstler JG, Ros R, Bittinger F, Metz T, Gebhard S, et al. c-erbB-2 expression in small-cell lung cancer is associated with poor prognosis. Int J Cancer. 2001;92:474-9.

14.     Potti A, Willardson J, Forseen C, Kishor Ganti A, Koch M, Hebert B, et al. Predictive role of HER-2/neu overexpression and clinical features at initial presentation in patients with extensive stage small cell lung carcinoma. Lung Cancer. 2002;36:257-61.

15.     Liu L, Shao X, Gao W, Bai J, Wang R, Huang P, et al. The role of human epidermal growth factor receptor 2 as a prognostic factor in lung cancer: a meta-analysis of published data. J Thorac Oncol. 2010;5:1922-32.

16.     Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136:215-33.

17.     Scott GK, Goga A, Bhaumik D, Berger CE, Sullivan CS, Benz CC. Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. J Biol Chem. 2007;282:1479-86.

18.     Nishida N, Mimori K, Fabbri M, Yokobori T, Sudo T, Tanaka F, et al. MicroRNA-125a-5p is an independent prognostic factor in gastric cancer and inhibits the proliferation of human gastric cancer cells in combination with trastuzumab. Clin Cancer Res. 2011;17:2725-33.

19.     Nakadate Y, Kodera Y, Kitamura Y, Shirasawa S, Tachibana T, Tamura T, et al. KRAS mutation confers resistance to antibody-dependent cellular cytotoxicity of cetuximab against human colorectal cancer cells. Int J Cancer. 2014;134:2146-55.

20.     Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105:10513-8.

21.     Kosaka N, Iguchi H, Yoshioka Y, Hagiwara K, Takeshita F, Ochiya T. Competitive interactions of cancer cells and normal cells via secretory microRNAs. J Biol Chem. 2012;287:1397-405.

22.     Onn A, Isobe T, Itasaka S, Wu W, O'Reilly MS, Ki Hong W, et al. Development of an orthotopic model to study the biology and therapy of primary human lung cancer in nude mice. Clin Cancer Res. 2003;9:5532-9.

23.     Sumikawa T, Shigeoka Y, Igishi T, Suyama H, Yamasaki A, Hashimoto K, et al. Dexamethasone interferes with trastuzumab-induced cell growth inhibition through restoration of AKT activity in BT-474 breast cancer cells. Int J Oncol. 2008;32:683-8.

24.     Minami T, Kijima T, Kohmo S, Arase H, Otani Y, Nagatomo I, et al. Overcoming chemoresistance of small-cell lung cancer through stepwise HER2-targeted antibody-dependent cell-mediated cytotoxicity and VEGF-targeted antiangiogenesis. Sci Rep. 2013;3:2669.

25.     Minami T, Kijima T, Otani Y, Kohmo S, Takahashi R, Nagatomo I, et al. HER2 as therapeutic target for overcoming ATP-binding cassette transporter-mediated chemoresistance in small cell lung cancer. Mol Cancer Ther. 2012;11:830-41.

26.     Rajabi H, Jin C, Ahmad R, McClary C, Joshi MD, Kufe D. MUCIN 1 ONCOPROTEIN EXPRESSION IS SUPPRESSED BY THE miR-125b ONCOMIR. Genes Cancer. 2010;1:62-8.

27.     Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, et al. Modulation of miR-155 and miR-125b Levels following Lipopolysaccharide/TNF- Stimulation and Their Possible Roles in Regulating the Response to Endotoxin Shock. The Journal of Immunology. 2007;179:5082-9.

28.     Zhou R, Hu G, Liu J, Gong AY, Drescher KM, Chen XM. NF-kappaB p65-dependent transactivation of miRNA genes following Cryptosporidium parvum infection stimulates epithelial cell immune responses. PLoS Pathog. 2009;5:e1000681.

29.     Kim SB, Kim JS, Lee JH, Yoon WJ, Lee DS, Ko MS, et al. NF-kappaB activation is required for cisplatin-induced apoptosis in head and neck squamous carcinoma cells. FEBS Lett. 2006;580:311-8.

30.     Lagadec P, Griessinger E, Nawrot MP, Fenouille N, Colosetti P, Imbert V, et al. Pharmacological targeting of NF-kappaB potentiates the effect of the topoisomerase inhibitor CPT-11 on colon cancer cells. Br J Cancer. 2008;98:335-44.

31.     Mendoza FJ, Ishdorj G, Hu X, Gibson SB. Death receptor-4 (DR4) expression is regulated by transcription factor NF-kappaB in response to etoposide treatment. Apoptosis. 2008;13:756-70.

32.     Spector NL, Blackwell KL. Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer. J Clin Oncol. 2009;27:5838-47.

33.     Naruse I, Fukumoto H, Saijo N, Nishio K. Enhanced anti-tumor effect of trastuzumab in combination with cisplatin. Jpn J Cancer Res. 2002;93:574-81.

34.     Kimura H, Sakai K, Arao T, Shimoyama T, Tamura T, Nishio K. Antibody-dependent cellular cytotoxicity of cetuximab against tumor cells with wild-type or mutant epidermal growth factor receptor. Cancer Sci. 2007;98:1275-80.

35.     Fukai J, Nishio K, Itakura T, Koizumi F. Antitumor activity of cetuximab against malignant glioma cells overexpressing EGFR deletion mutant variant III. Cancer Sci. 2008;99:2062-9.

36.     Nakadate Y, Kodera Y, Kitamura Y, Shirasawa S, Tachibana T, Tamura T, et al. KRAS mutation confers resistance to antibody-dependent cellular cytotoxity of cetuximab against human colorectal cancer cells. Int J Cancer. 2013.

37.     Tamura K, Shimizu C, Hojo T, Akashi-Tanaka S, Kinoshita T, Yonemori K, et al. FcgammaR2A and 3A polymorphisms predict clinical outcome of trastuzumab in both neoadjuvant and metastatic settings in patients with HER2-positive breast cancer. Ann Oncol. 2011;22:1302-7.

**Figure legends.**

**Figure 1. HER2 expression in various SCLC cell lines.**

(A) Western blotting (WB) analysis of HER family expression in SCLC cell lines using breast cancer cell lines as positive controls. (B) Flowcytometry (FACS) analysis of HER2 expression in SCLC and breast cancer cell lines. Both assays indicated that, of the SCLC cell lines analyzed, HER2 was expressed in SBC-3 and SBC-5 cell lines.

**Figure 2. HER2 expression change after exposure of SCLC cells to CDDP.**

HER2-positive SCLC cells were exposed to CDDP at the $IC_{50}$ concentration for each cell line. HER2 expression was then analyzed by (A) WB analysis after exposure of the cells to CDDP for up to 48 hr; (B) qRT-PCR analysis and (C) FACS analysis. Expression change of miR-125a and miR-125b in the cells was also analyzed using qRT-PCR (D). Experiments were repeated at least three times with similar results. Error bars represent the mean ± SD. *$p<0.05$. NC, negative control.

**Figure 3. HER2 expression is directly regulated by microRNA-125a and microRNA-125b.**

(A) Scheme of the putative target sites of miR-125a and miR-125b in the 3'UTR of HER2 mRNA and the sequence of the mutant UTR. (B) The effect of co-transfection of pre-miR-125a or pre-miR-125b with wild-type or mutant 3'UTR reporter vectors in SBC-5

cells was measured using luciferase reporter assays. (C) qRT-PCR analyses of HER2 gene expression after treatment of SBC-3 and SBC-5 cells with pre-miR-125a, pre-miR-125b, anti-miR-125a, anti-miR-125b or control. (D) WB analyses of HER2 after treatment of SBC-3 and SBC-5 cells with pre-miR-125a, pre-miR-125b, anti-miR-125a or anti-miR-125b. All the experiments were repeated at least three times with similar results, and error bars represent the mean ± SD. *$P<0.05$. NC, negative control.

**Figure 4. ADCC against HER2-positive SCLC cells mediated by trastuzumab and NK92/CD16a.**

(A) Cell viability of SBC-3 and SBC-5 cells was determined 72 hr after treatment with CDDP ($IC_{50}$ dose for each cell type), trastuzumab (1 μg/ml) or CDDP plus trastuzumab in the presence or absence of effector cells using a WST-8 assay. NK92/CD16a cells were used as effector cells at an E:T ratio of 1:1. (B) The target cells were incubated with the effector cells at an E:T ratio of 10:1, along with various concentrations of trastuzumab, for 4 hr. ADCC was determined using a calcein assay. (C) Calcein assay with various E (NK92/CD16a):T ratios. (D) Calcein assay of HER2-positive SCLC cells after 48 h exposure to CDDP ($IC_{50}$ dose for each cell type) and subsequent trastuzumab (1 μg/ml) treatment at an E:T ratio of 10:1 for 4 hr. All experiments were repeated at least three times with similar results. Error bars represent the mean ± SD. *$P<0.05$. NC, negative control.

**Figure 5. An *in vivo* study of the enhanced anti-tumor effect of trastuzumab and CDDP.**

(A) The protocol of the *in vivo* study. Mice were injected with $1\times10^{6}$ SBC-5-Luc cells into the left lung on day 0. Each treatment: CDDP (3 mg/kg) alone; trastuzumab (20 mg/kg) alone or CDDP (3 mg/kg) plus trastuzumab (20 mg/kg), was performed on days 7, 14, 21 and 28 (once a week for 4 weeks, four treatments in total). (B) Bioluminescence imaging was quantified and is expressed as relative luminescent intensity (luminescent intensity on day 7 in each group was considered as 100). (C) Change in the bioluminescence emitted from the whole bodies of the mice in each treatment group. *$P<0.05$.

**Figure1**

Figure2

**A**



**B**



**C**



| | Percent Gated |
|---|---|
| isotype control | 0.00% |
| Control | 0.29% |
| CDDP 24hr | 16.02% |
| CDDP 48hr | 56.27% |

| | Percent Gated |
|---|---|
| isotype control | 0.00% |
| Control | 8.80% |
| CDDP 24hr | 39.46% |
| CDDP 48hr | 82.72% |

**D**

# Figure3

**A**

Position 37-43 of
HER2 3'-UTR;

5'-...GAUGUGUC**CUCAGGG**A...-3'

| | | | | | |

hsa-miR-125a;  3'- AGUGUCCAAUUUCCCA**GA GUCCC**U-5'

hsa-miR-125b;  3'- AGUGUUCAAUCCCA**GAG UCCC**U-5'

| | | | | |

mutated UTR;  5'- ...**CACTGCG**...-3'

**B**



**C**



**D**

Figure4

**Figure5**

**A**

Group 1 (control)

Group 2 (CDDP)

Group 3 (Trastuzumab)

Group 4 (CDDP+Trastuzumab)

Days    0    7    14    21    28    35    42

Evaluation by IVIS imaging

Intrathoracic injection (left lung) of SBC-5-Luc ($1\times10^6$ cells)

▼ CDDP treatment: 3 mg/kg, i.p.

⬇ Trastuzumab treatment: 20 mg/kg, i.p.

**B**

Relative bioluminescence intensity

control
CDDP
Trastuzumab
CDDP+Trastuzumab

day7  day14  day21  day28  day35  day42

**C**

control    CDDP    Trastuzumab    CDDP+Trastuzumab

Day7

Day21

Day42

$5\times10^5$  $1.5\times10^4$ (photons/s)

$1\times10^6$  $5\times10^4$ (photons/s)

$2\times10^5$  $2\times10^4$ (photons/s)

$2\times10^5$  $1.5\times10^4$ (photons/s)
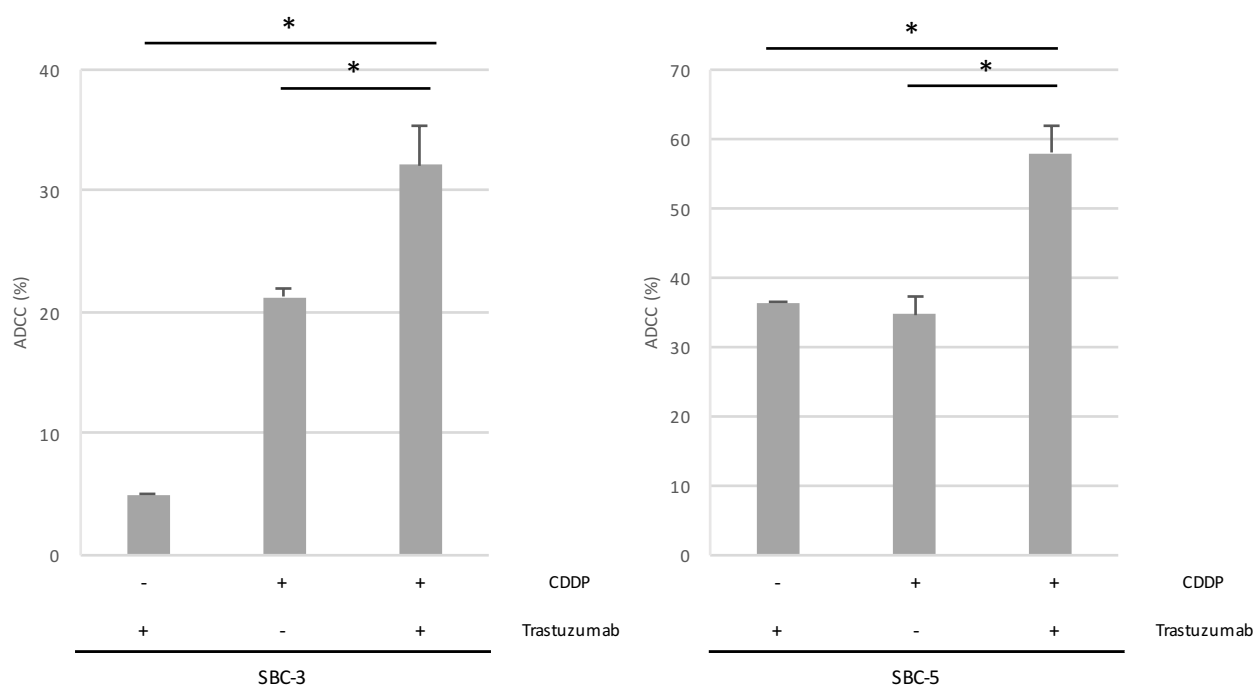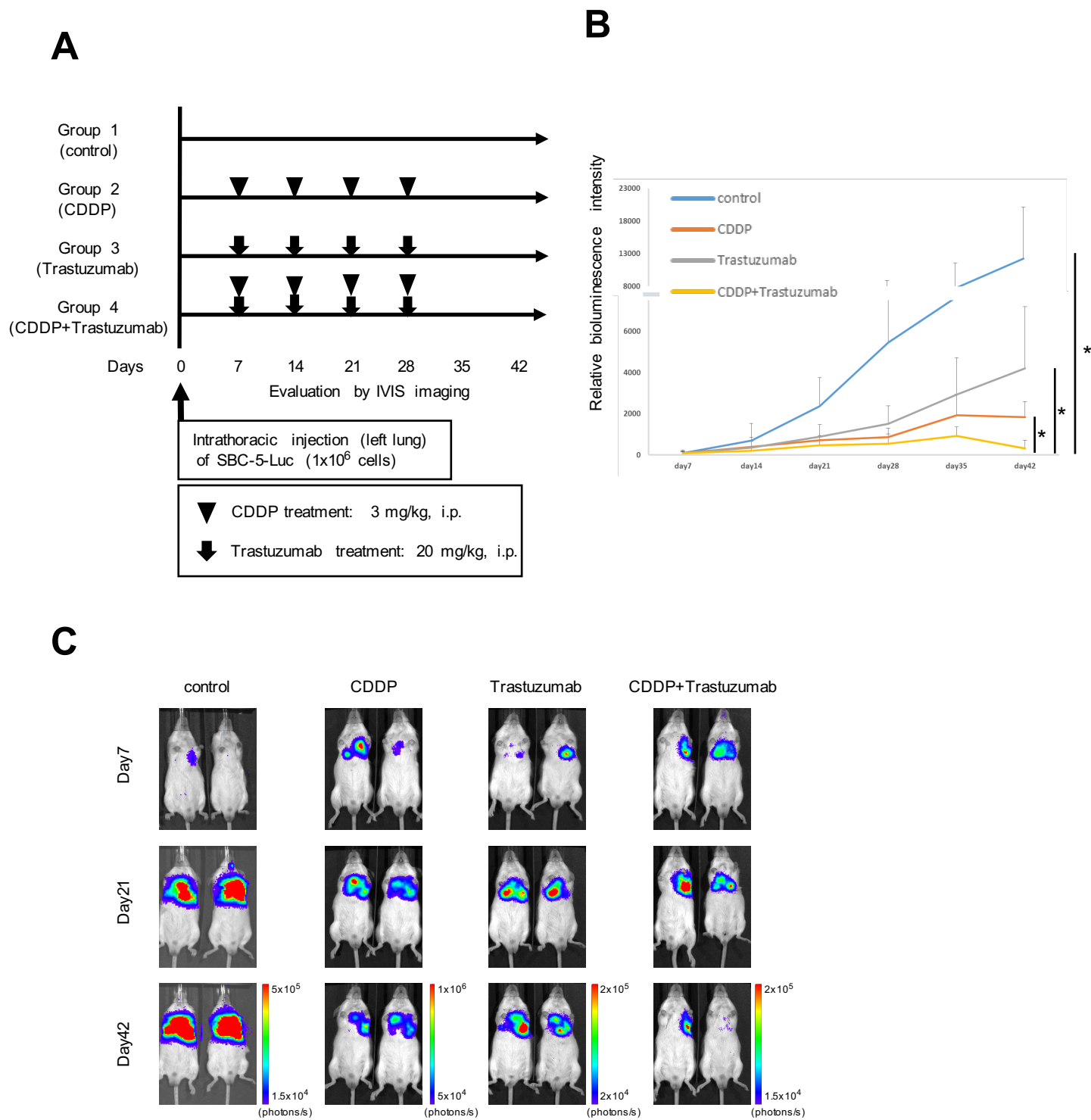
# Supplementary Information

**Chemotherapy regulated microRNA-125/HER2 pathway as a novel therapeutic target for trastuzumab-mediated cellular cytotoxicity in small-cell lung cancer.**

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Supplementary Figures 1-2
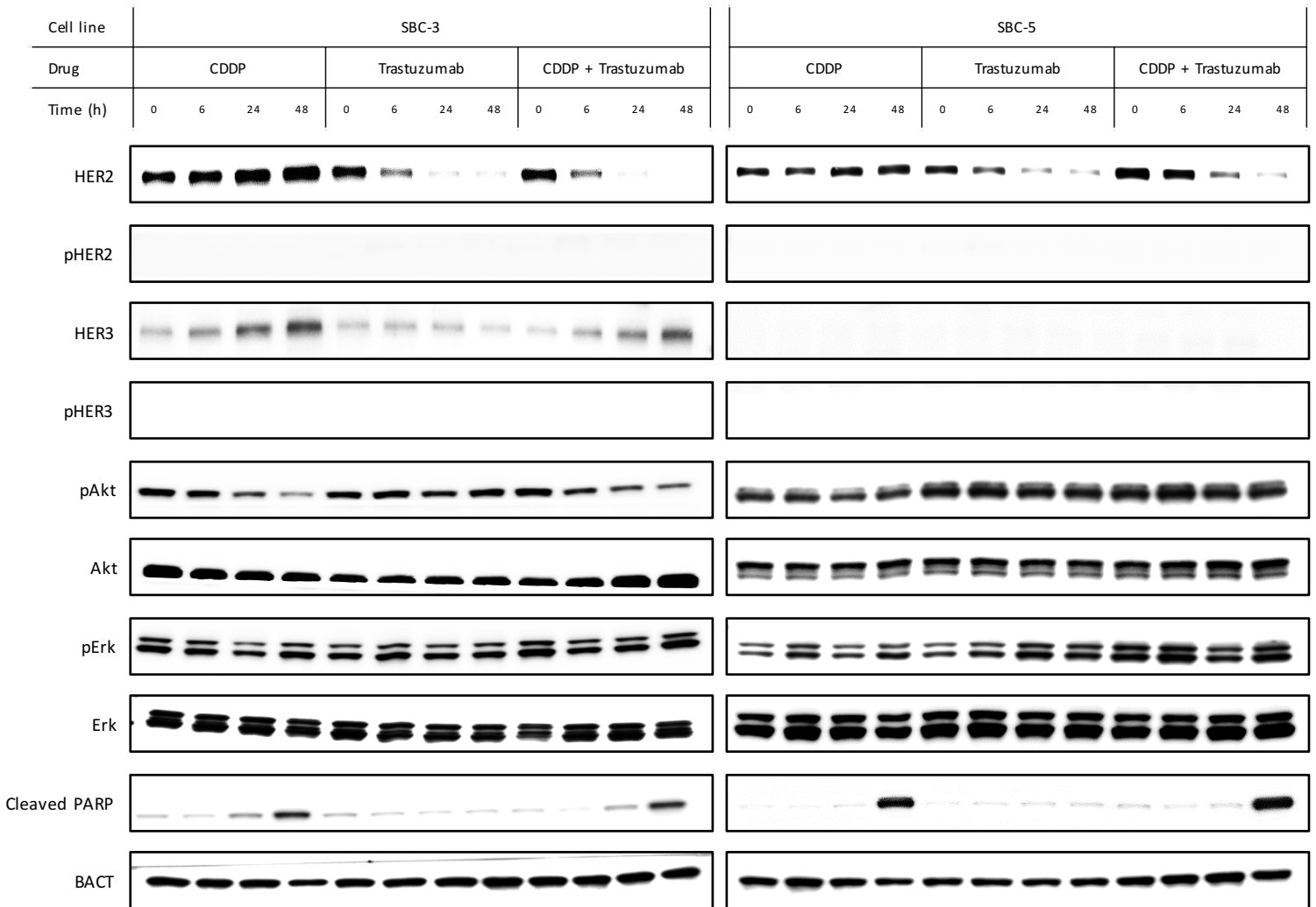Supplementary Tables 1-2

## A



## B
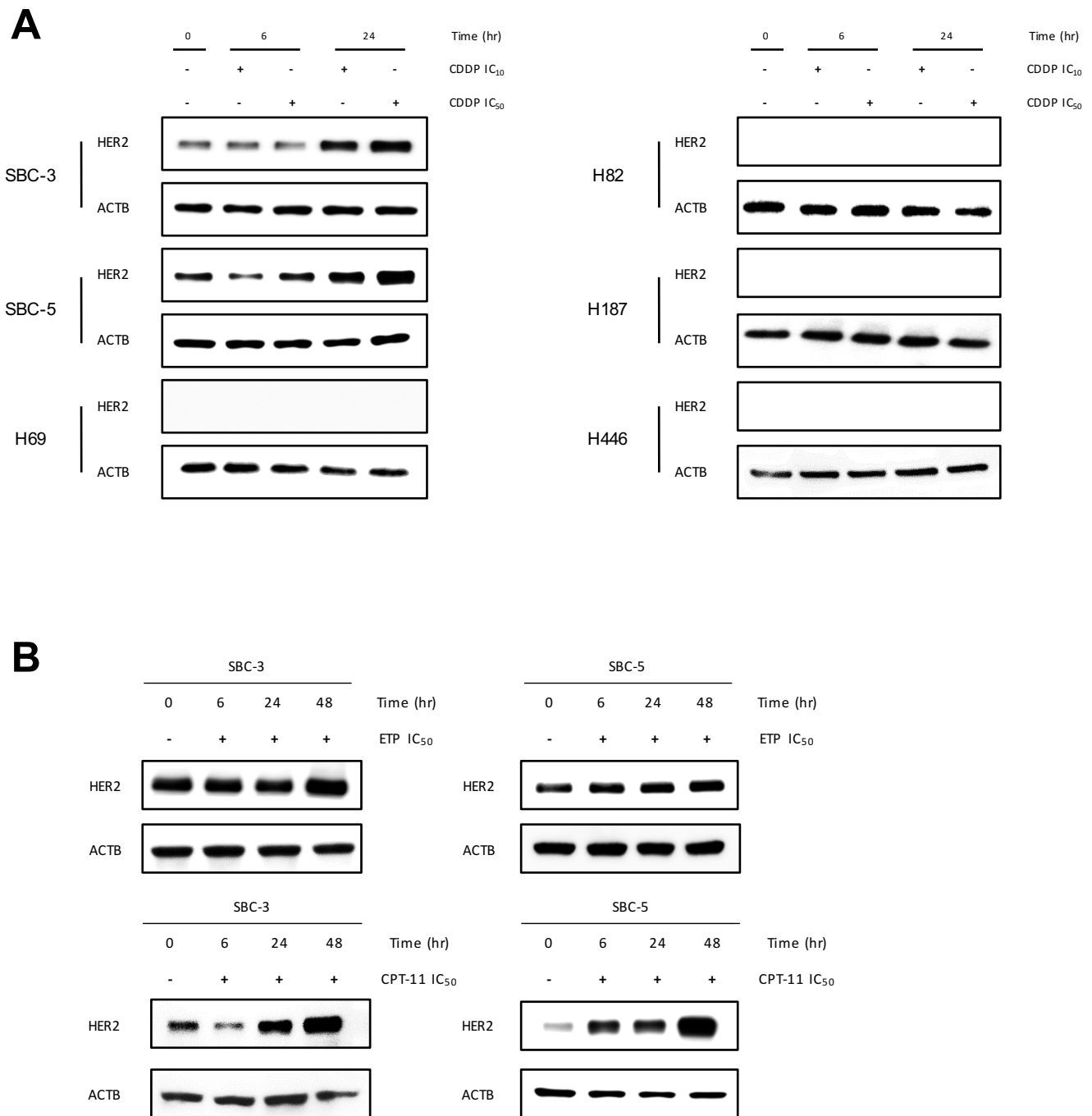


**Supplementary Figure 1. HER2 expression change after exposure of SCLC cells to various cytotoxic drugs.**

(A) WB analysis of HER2 expression after exposure of the indicated SCLC cells to CDDP at $IC_{10}$ and $IC_{50}$ concentrations for the indicated times. (B) WB analysis of HER2 expression after exposure of SBC-3 and SBC-5 cells to ETP and CPT-11 at their $IC_{50}$ concentrations. All experiments were repeated at least three times with similar results. ETP: etoposide, CPT-11: irinotecan.

**Supplementary Figure 2. Intracellular signaling after drug exposure.**

WB analysis for downstream intracellular signaling of HER2 after exposure to CDDP ($IC_{50}$ concentration), trastuzumab (1 μg/ml), and CDDP plus trastuzumab.

Supplementary Table 1. Inhibitory concentration of 50% dose

| Cell line | $IC_{50} \pm SD$ (μM) |
| --- | --- |
| SBC-3 | 1.46 ± 0.35 |
| SBC-5 | 2.06 ± 0.06 |
| H69 | 5.10 ± 0.13 |
| H82 | 0.40 ± 0.10 |
| H187 | 0.52 ± 0.03 |
| H446 | 6.40 ± 0.30 |

Supplementary Table 2. The primer sequences for qRT-PCR analysis

| Gene | Forward primer (5'-Sequence-3') | Reverse primer (5'-Sequence-3') |
|------|----------------------------------|----------------------------------|
| HER2 | AAAACACATCCCCCAAAGC | AGGGCATAAGCTGTGTCACC |
| ACTB | ATTGCCGACAGGATGCAGA | GAGTACTTGCGCTCAGGAGGA |